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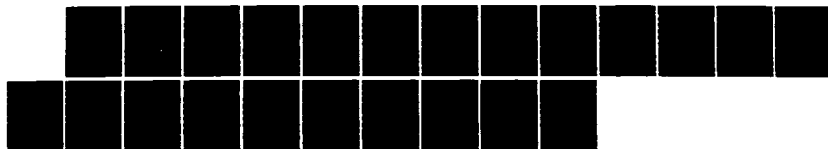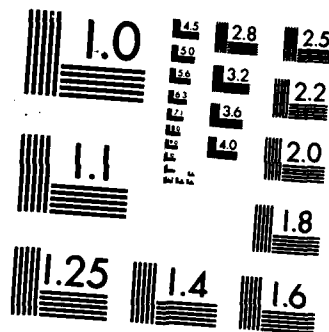   DEPT OF BIOCHEMISTRY   R K BROWN 01 AUG 86
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Type A human red blood cells, alpha-N-acetylgalactosaminidase, affinity chromatography, radioimmunoassay, enzyme purification, conversion to O cells, human placenta, Clostridium paraputrificum

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

N-acetylgalactosaminidases, enzymes which convert type A human red blood cells to type O, have been purified from human placenta and from culture medium of Clostridium paraputrificum. Their properties have been studied and their ability to remove type A determinants has been quantitated by radioimmunoassay.

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PURIFICATION OF N-ACETYLGALACTOSAMINIDASE

BY ISOELECTRIC FOCUSING

Contract No. N 00014-83-K-0339

Final Report

for the period

April 15, 1983 thru July 15, 1986

Ray. K. Brown
Principal Investigator

Department of Biochemistry
Wayne State University School of Medicine
Detroit, MI 48201

QUALITY
INSPECTED
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This is the final technical report on Contract N 00014-83-K-0339. This project was directed at obtaining and purifying enzymes which could convert type A human red blood cells to type O. The latter can be given to all recipients and the availability of such cells would simplify inventory and cross matching during emergencies. Chemically the A antigens differ from the O antigens in having an additional terminal sugar residue, an α linked N-acetyl- galactosamine. Several enzymes have been studied which remove this residue and convert A substance to O substance. These enzymes, α-N-acetyl-galactosaminidases, are referred to as Azymes in this report. Related work on the isoelectric focusing of Azyme from <u>Clostridium perfringens</u> and the purification and assay of Azyme from human placenta has been described in our final report on Contract N-00014-78C-0767, Task No. NR 207-143, AD number A 154749. Several areas are covered in more detail in the present report than may be useful for some readers. These paragraphs are indented and may be elided without loss of continuity. The introduction and materials and methods sections may also be skipped over by those interested only in the results of the contract.

INTRODUCTION
The A antigens of red blood cells are complex mixtures of substances. Each determinant however contains the same three sugars in the same linkages (galNAc $\alpha 1 \rightarrow 3$-[Fuc $\alpha 1 \rightarrow 2$]gal) attached through other sugars. In erythro-cytes both membrane proteins and glycolipids carry type A blood group determinants. The major proteins are band 3 and band 4.5 glycoproteins. The glycolipids have been most extensively studied. Until recently there were four major classes of A substance generally recognized. During the past two years several additional types of determinants have been delineated. Hakomori and his coworkers have been especially active in this area. Figure 1 (Clausen et al 1985a) illustrates some of this diversity. The wide variety of A determinants may be related to the difficulties in removing the last traces of A activity from treated cells.

The specificity of monoclonal antibodies reacting with type A determinants for different portions of the site has been studied by three groups of investigators, Chen and Kabat (1985), Gooi et al (1985) and Furukawa et al (1985). Several of the antibodies combine with all type A determinants. Other antibodies react only with difucosyl determinants or with monofucosyl groups regardless of the type of chain to which they are attached. Some are chain specific (Clausen et al, 1985b, Furukawa et al, 1985). Specific antibodies for types 1, difucosyl type 2 and type 3 chains have been found. An antibody of this type has been used to probe the difference between A_1 and A_2 cells. A_1 cells have some A antigen with a repetitive structure not found in A_2 cells (Clausen et al 1985c). It has been possible to completely convert A_2 cells to O with the enzymes tested in this report while some residual A activity always remains with treated A_1 cells. The cross reactions of such antibodies have also been studied and are of especial interest in certain cancer groups (Hirohashi et al, 1985). The monoclonal antibody used in the present studies was among those studied by Furukawa et al (1985). It is antibody CB which reacted with all of the A varient structures tested. It was obtained commercially and was made using a synthetic antigen.
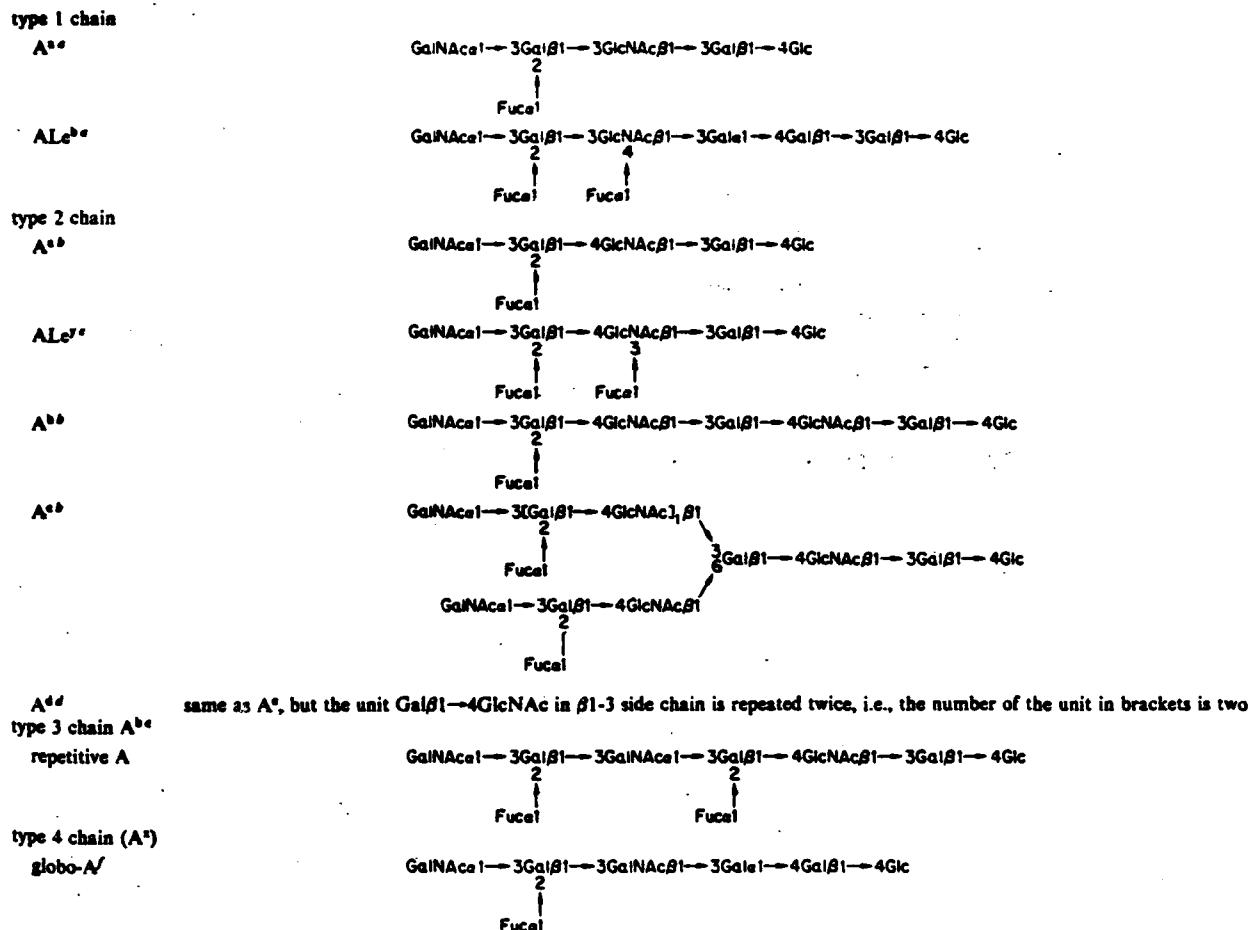
type 1 chain

A$^{a·}$ GalNAcα1 → 3Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4Glc
                           2
                           ↑
                         Fucα1

ALe$^{b·}$ GalNAcα1 → 3Galβ1 → 3GlcNAcβ1 → 3Galα1 → 4Galβ1 → 3Galβ1 → 4Glc
                        2   4
                        ↑   ↑
                       Fucα1   Fucα1

type 2 chain

A$^{a·b}$ GalNAcα1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc
                        2
                        ↑
                     Fucα1

ALe$^{y·}$ GalNAcα1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc
                        2   3
                        ↑   ↑
                     Fucα1   Fucα1

A$^{b·b}$ GalNAcα1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc
                        2
                        ↑
                     Fucα1

A$^{c·b}$ GalNAcα1 → 3[Galβ1 → 4GlcNAc]₁ β1
                        2   ↘
                        ↑   3
                     Fucα1   6 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc
                                           ↗
                    GalNAcα1 → 3Galβ1 → 4GlcNAcβ1
                              2
                              ↑
                         Fucα1

A$^{d·d}$ same as A^c, but the unit Galβ1 → 4GlcNAc in β1-3 side chain is repeated twice, i.e., the number of the unit in brackets is two

type 3 chain A$^{b·}$

    repetitive A   GalNAcα1 → 3Galβ1 → 3GalNAcα1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc
                                 2   2
                                 ↑   ↑
                             Fucα1   Fucα1

type 4 chain (A^z)

    globo-A^f GalNAcα1 → 3Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc
                            2
                            ↑
                       Fucα1

Fig. 1   Type A Determinants of Human Erythrocytes (Clausen et al, 1985a)

MATERIALS AND METHODS

Affinity matrix

The affinity agent used was originally developed by Harpaz, Flowers and Sharon (1974) for the purification of coffee bean alpha-galactosidase. It was used by Bishop and Desnick (1981) for the preparation of alpha galactosidase from human spleen, placenta and plasma. During that work they found that Azyme could also be purified with this agent but they did not study the conditions or enzyme further. The synthesis of the affinity matrix is outlined in the following indented paragraphs.

> Synthesis of N-benzyloxy carbonyl-6-amino hexanoic acid (3) was by the method of Schwyzer et al (1962). Benzyl chloroformate (2) (150.65 g in 300 ml of diethyl ether) was added simultaneously with an equal volume of 4 N NaOH containing 3.11 g of 6-amino hexanoic acid (1). The mixture was stirred for 1 hr at room temperature and extracted 3 times each with 300 ml of ether. The aqueous phase was brought to pH 2.0 with 6 N HCl and stored overnight at 4°. The precipitate was removed by filtration and dried under vacuum. The precipitate was crystallized twice from CCl₄ at 4° yielding 1.85 g of product with a melting point of 54-55.2°. The material of Schwyzer melted at 53-55°. The yield was 35%. Four similar batches were made yielding in all 8.86 g of product.
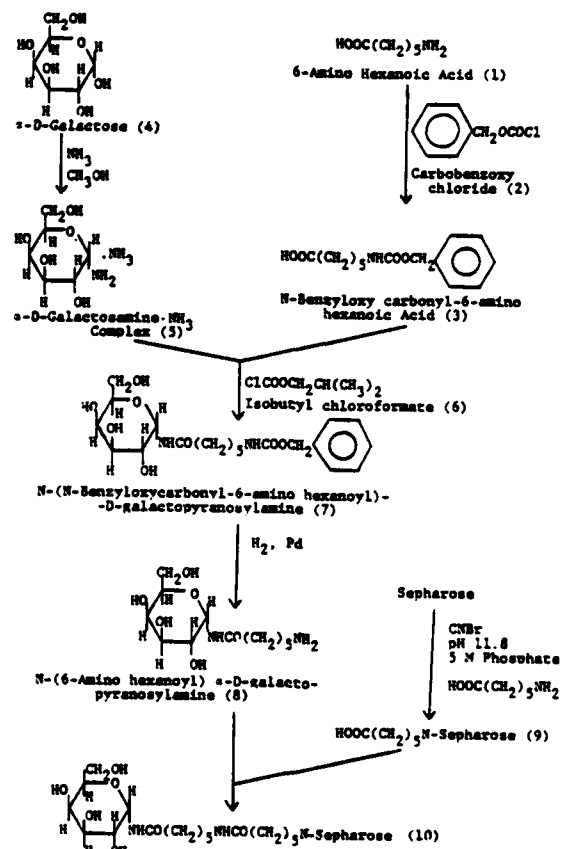
Fig. 2 Synthesis of Affinity Support

Synthesis of α-D-galactosamine.NH_3 complex (5) was by the procedure of Frush and Isbell (1951). Galactose (4) (39.64g) was partially dissolved in 100 ml of absolute methanol containing 1.02 g of NH_4Cl. Ammonia gas was bubbled through the material for 7 hr at 0°. After storage for 2 days at 4°, the precipitate was collected by filtration and washed with 800 ml of absolute methanol at 4°. The ammonia saturated methanol was prepared by bubbling ammonia through methanol cooled in a dry ice/ethanol bath for 2 hrs. The washed precipitate was dissolved in 200 ml of 3% ammonium hydroxide and crystallized by the addition of ammonia saturated methanol. The material was crystallized an additional 4 times and yielded 5.72 g of material which had an $[\alpha]_D$ at 23° of 108–111°. Harpaz et al obtained a value of 107–109°. It was essential to keep the derivative under ammonia at all times.

N-(N-Benzyloxycarbonyl-6-amino hexanoyl- α -D-galactopyranosyl-amine (7) was made by the procedure fo Harpaz et al. Isobutyl chloroformate (6) (3.9 ml) and triethylamine (4.1 ml) were simultaneously added to a stirred precooled solution of N-benzyloxy-6-amino hexanoic acid (7.95g) in 50 ml of dry dimethyl formamide. After stirring for 20 min at -5°, the mixture was filtered and the filtrate added to 2.45 g of the α-D-galacto-pyranosylamine-ammonia complex in 50 ml of dry dimethylformamide. After stirring for 20 hrs at 4° the sample was filtered at room temperature and the filtrate evaporated to yield 11.85 g of syrup. This was dissolved in 20 ml of chloroform: methanol (2:1) and applied to a 5.5x97 cm column of Silica Gel H (Sigma). The column was developed at a flow rate of 30 ml/hr.

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Twenty ml fractions were collected and every fifth fraction was tested with orcinol reagent. Fractions 145-180 were concentrated by evaporation. After drying twice from chloroform:methanol (2:1), 2.77g of material was obtained. The carbobenzyloxy group was removed by hydrogenation. For this a hydrogenation apparatus similar to that described by Vogel (1956) was constructed and tested by reducing maleic acid to succinic acid using a catalyst containing 10% palladium on charcoal. The hydrogen uptake was 14.6% greater than theory possibly due to a small leak. For the hydrogenation 2.77 g of the carbobenzoxy derivative (7) was dissolved in 100 ml of 80% aqueous methanol and filtered. Catalyst (0.1 g) was added and the hydrogenation allowed to proceed for 22 hr. The uptake was 170 ml (theory 157 ml). The product, N-6-aminohexanoyl-α-D-galactopyranosylamine (8) (1.68 g)(90% yield) was dried by flash evaporation and stored over $CaCl_2$.

Carboxyhexyl-Sepharose (9) was prepared by the procedure of Porath et al (1973). Sepharose 4B (100 ml) was suspended in 100 ml of 5 M phosphate buffer at pH 11.8 and cooled to 10°. A solution containing 2 g of cyanogen bromide, 10 ml of water and 1 ml of acetonitrile was added slowly. After 20 min the Sepharose was washed on a sintered glass filter with 500 ml of 0.1 M sodium bicarbonate buffer at pH 9.5. The Sepharose was added to 100 ml of the buffer containing 26.24 g of 6-aminohexanoic acid and stirred slowly at room temperature for 3 hr and then stored for 16 hr at 4°. Ethanolamine (9.16 g) was added and the suspension stirred for 3 hr. The beads were washed with 400 ml of water followed by 200 ml of 0,1 N HCl which was slowly percolated through the beads. The material was then washed with 400 ml of water followed by 200 ml of 0.1 N HCl. The material was then washed with 400 ml of 0.2 M KCl and stored in 0.2 M KCl containing 0.02% sodium azide.

The N-6-aminohexanoyl-α-D-galactopyranosylamine (8) was coupled to the carboxyhexyl-Sepharose by the method of Bishop and Desnick (1981). The carboxyhexyl-Sepharose (25 ml of packed gel) was filtered to near dryness and coupled with 1.68 g of the hexanoyl galactosamine in 100 ml of water at room temperature. Five g of 1 ethyl-3-(3 dimethylpropyl)-carbodiimide HCl was added initially and again after 1/2 and 1 hr. The material was allowed to stand for 20 hr and then 7.33 g of ethanolamine was reacted with the beads at room temperature. The filtrate and first water wash were saved and concentrated to 30 ml for further coupling. The beads were washed with 400 ml of water and stored in 50 ml of water containing 0.02% sodium azide. All containers coming in contact with the Sepharose during the coupling were siliconized prior to use. All of the first preparation of affinity matrix was stored at 4° in 0.02% sodium azide and became inactive after about 8 months. A second lot was made and about half was freeze dried for storage with dessicant at -20°. It appears stable under these conditions.

Preliminary tests were done with Azyme from a DEAE fraction from human placenta, from the gel filtration step of a clostridial preparation and from partially purified material from Charonia lampas. All were absorbed. The placental enzyme was absorbed from citrate phosphate buffer pH 4.7 containing 26.7mM citric acid, 46.6mM Na_2PO_4 and 150mM NaCl. For the clostridial enzyme the buffer was 0.01 M phosphate pH 7.0 containing 0.01mM DTT. The Charonia lampas enzyme was absorbed from pH 4.0 citrate-phosphate containing 30.7mM

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citric acid, 38.6mM Na$_2$PO$_4$ and 500mM NaCl. These conditions were chosen because they are those used for assay and accordingly those where one would expect a high interaction with substrate.

Radioimmunoassay

Monoclonal antibodies (Lots AH7-156, AH7-216, AH8-39, and AH4-14) obtained from Chembiomed Ltd. (Edmonton, Alberta, Canada) were used in the radioimmunoassays described here. The antibody was prepared to the terminal trisaccharide of the group A determinant coupled to albumin. Specifically purified antibody was used. Our earlier work employed monoclonal antibodies prepared here in collaboration with Dr. Myron Leon of the Department of Immunology and Microbiology. These radioimmunoassays had a larger than desirable background when type O cells were used. It appears that much of this was due to the iodination procedure. In the next experiments the method of Tejedor and Ballesta (1982) was used. This system keeps the iodine generating reaction and the labeling in separate phases. Chloroamine T is used to generate Cl$_2$ from a circle of Whatman 3MM filter paper which had been impregnated with 1 M NaCl. It was placed over the sample which was in 50 ul of pH 7.5 phosphate buffer (0.05 M). ^{125}I(100 uCi) was mixed with the sample. The reaction was carried out in the bottom of a 5 ml polypropylene centrifuge tube which had been cut from the remainder of the tube. It was important to have the paper near the reaction mixture. A 2.4 cm tube gave 1.4% incorporation into the antibody while a 0.6 cm tube gave a 17.4% incoporation. The paper was replaced with a fresh piece at 10 min intervals. Four papers were used. The labeled antibody was separated from residual ^{125}I by collecting 300 ul fractions from a 1.0x15 cm column of Sephadex G25 which had been equilibrated with 150 mM NaCl containing 25 mM phosphate buffer pH 7.5 and 50 ug of hen egg white lysozyme/ml. Although the antibody labeled in this manner worked well, the procedure was somewhat cumbersome. Recently labeling has been done using Protag (J.T. Baker Chemical Co.). A typical protocol follows: sodium borate buffer (10 mM borate, 150 mM NaCl pH 8.2), 200 ul, was added to 26 ul of antibody (0.1mg) and 5X10^7 cpm of carrier free Na^{125}I in a 0.5 ml poylethylene centrifuge tube. Its contents were cooled to 0° and then 25 mg of Protag was added and the reaction was mixed on a vortex mixer. After 10 minutes in an ice bath, the supernatant was removed and desalted as above. Under assay conditions 32-36% of counts bound to A cells and 1.2% to O cells. The binding was completely inhibited by A substance indicating that it was specific (Fig. 3).
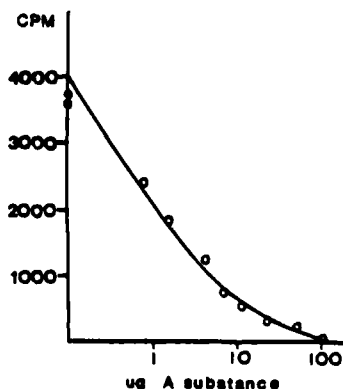

Fig. 3 Inhibition of binding of monoclonal antibody by group A substance

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For the assay fresh A and O cells from the Southeast Michigan Red Cross were used. They were from the pilot tubes drawn to check for hepatitis etc. Most experiments were done with cells drawn 2-5 days previously. The Azyme for the experiment was dialyzed against the appropriate buffer overnight before use. It takes about .01 unit of the placental Azyme (ie. the amount of enzyme that would liberate .01 umole of nitrophenol/min) for a determination using 125 ul of packed red blood cells. Experiments with the clostridial Azyme used 0.02 to 0.08 units. The Azyme was in a final volume of 100 ul. Since there are volume changes during dialysis, a more concentrated enzyme solution was used and brought to the final volume with washings of the dialysis bag. For the experiments with the clostridial enzyme a 50% cell suspension was used and duplicate 10 ul aliqouts were diluted to 50 ul and 12.5 ul samples of this were assayed. Plastic microfuge tubes were used for containers. The cells were tested before use to be sure that they were correctly typed. The cells were washed 3 times with the buffer taking care to remove the buffy coat. The cells were centrifuged for 10 min after the last wash to pack them reproducably. The assay was for 10 ul of a 50% cell suspension. It was added to 115 ul of buffer. An amount of diluted labeled antibody sufficient to contain about 20,000 cmp (10-25 ul) was added to 12.5 ul of the diluted red cell mixture. There was a linear relationship between the counts bound and the number of A cells whether they were used alone or with appropriate amounts of O cells to keep the total number of cells constant. The results were independent of volume between 11 and 25 ul with a 13% decrease at 100 ul. It · was done in the 0.5 ml plastic centrifuge tubes. The red cell suspensions were well mixed just before pipeting as they settled out quite rapidly. The ^{125}I pipetting was quite critical. The pipet was rinsed a few times in the ^{125}I labeled antibody before starting. Counts adhered to the plastic of the tip and, if it wasn't rinsed first, the first determination of a series did not have as many input counts. After adding the ^{125}I antibody the tubes were mixed on a Vortex mixer at once and incubated at 37° in a water bath for 30 min., removed and input counts done. PBS (0.5 ml) was added. When a smaller wash volume is used the background counts increase. Typical experiments had background counts of 43 for O cells compared to 9099 for A cells (0.47%) and 89/10123 (0.88%). It was important not to vortex the washes because cells got upon the walls of the tube. The tubes were centrifuged 3 min in an Eppendorf centrifuge in the cold room. The supernatant was removed leaving a little (eg. 10-20 ul)over the red cells. The washing procedure was repeated 3 more times. After the final super had been removed, the cells were quantitatively transferred to a fresh tube because the old tube had some counts, usually about 25% of the input counts, adsorbed to it. Unlabeled monoclonal antibody displaced the labeled antibody. Five ul of unlabeled antibody displaced 3258 counts in a system which contained 6 ul of labeled antibody (7944 bound counts).

Enzyme assays

The clostridial enzyme was assayed in 0.01 M sodium phosphate buffer pH 7.0 which was 0.1 mM in dithiothreitol and contained 0.02% sodium azide. The stock substrate was made in this buffer and contained 5 mg of p-nitrophenyl-2-acetamido-2-deoxy-α-D-galactosamine per 2.5 ml. The reaction mixture contained 22 ul of enzyme, 34 ul of substrate, and 6 ul of 7 mM $CaCl_2$. After incubation for 1 hr at 37°, the reaction was stopped with 400 ul of 0.3 M sodium carbonate and read at 400 nm. Blanks were incubations ran without substrate. There was no substrate hydrolysis under the assay conditions in the absence of

enzyme. Samples with high blanks were also assayed as required by adding 25ul of 50% trichloroacetic acid in 1 M NaCl after incubation. After centrifugation 43.5 ul of super were added to 75 ul of 2.0 N NaOH and 343.5 ul of 15% Na_2CO_3.

The placental Azyme activity was measured in a 250 ul volume containing 0.36 mM p-nitrophenyl-2-acetamido-2deoxy-α- D-galactopyranoside in 1 M sodium acetate buffer pH 4.0. After incubation for 1 hr at 37°, the reaction was terminated with 200 ul of 15% Na_2CO_3 and read at 400 nm. In some cases the assay was terminated with 100 ul of 50% TCA in 1 M NaCl. After centrifugation an aliquot of the supernatant (175ul) was removed, neutralized with 300 ul of 2 N NaOH and 140 ul of 15% Na_2CO_3 was added.

The enzyme activity is expressed as umoles of substrate hydrolyzed per minute using an extinction coefficient of 1.741×10^{-4} liters mol^{-1} cm^{-1}.

RESULTS
Culture of Clostridium paraputrificum

The blood group degrading activity of filtrates of <u>Clostridium paraputrificum</u> has been previously examined by Nicholas Paoni and his coworkers at the Naval Biosciences Laboratory in Oakland, CA. Cultures and samples of partially purified enzyme were obtained from Joan Quay at NBL. Dr. Bong Park, a Visiting Scientist, undertook the culture of the organism and studied the early steps in purification of the enzyme. He optimized the time and temperature and found that times longer than the 13 hr suggested by Paoni et al are better than shorter times. At high temperatures the organism sporulates and the spores are also a satisfactory innoculum. The organism is not harmed by less than rigorous anaerobisis. Cultures plugged with cotton or covered with loose fitting caps grow and produce well. He has shown that the bacterium grows and produces Azyme in a synthetic medium. The effect of medium concentration on Azyme production was examined. It was found that Azyme production is about twice as great at 2/3 the concentration of medium. This material also filters much more readily through the 0.45 u Millipore filter used to prepare the culture supernatant. The specific activity of Azyme from a 10 day culture was independent of the concentration of the medium although concentrations less than 1/2 the original gave decreased amounts of enzyme. The best culture pH is 5.6 (Table I).

Table I. Effect of pH of Culture

pH	5.6	6.23	7.4
U/ml	5.5×10^{-3}	2.7×10^{-3}	0.86×10^{-3}
SA	1.0×10^{-3}	0.56×10^{-3}	0.24×10^{-3}

An important aspect of the work with the growth of clostridia is the studies done with material grown in a defined medium. For this purpose the medium developed for <u>Neisseria gonorrhoeae</u> by Morse and Bartenstein (1980) was used. It is a mixture of salts including $CaCl_2$ and $Fe(NO_3)_3$, amino acids, thiamine, pantothenate, biotin, glucose, hypoxanthine and uracil. For Azyme production it is necessary to add 0.02% gastric mucin, the only undefined constituent.

The color found in culture filtrates is caused in part by materials reacting under the influence of light. Filtrates should be kept in the dark as much as

possible.

The present procedure is to prepare the following medium: Todd Hewitt Broth (Difco) 100 g, NaCl 25 g, K_2HPO_4 20 g, glucose 15 g, water to make 10 l. Medium is autoclaved at 121° and inoculated with C. paraputrificum which had been stored at -70° and preincubated overnight in 10 ml of broth at 37° in a capped 20 ml culture tube. The inocculum was added to the prewarmed medium and incubated for 48 hr at 37° without shaking.

Purification of clostridial Azyme

While fractionation with ammonium sulfate has been studied, for many experiments, the enzyme has been precipitated at 90% saturation to avoid any loss of enzyme in case more than a single activity occured. Later experiments showed that there were two enzymes which hydrolyzed the synthetic substrate but only one which attacked the A determinant on red cells. The current procedure is as follows. The crude enzyme was harvested by adding 5.16 Kg of ammonium sulfate to the culture and gently stirring overnight at 4°. The precipitate was separated by centrifuging for 30 min at 10,000 rpm in a Sorvall centrifuge using a GSA head (16,300 G). The precipitate was suspended in 400 ml of water containing 84 g of ammonium sulfate (35% saturation) and centrifuged 1 hr under the above conditions. The supernatant was filtered through a 0.45 um filter (Millipore), concentrated to about 20 ml by ultra-filtration (Amicon Model 402 filter) and dialyzed against water containing 0.02% sodium azide.The enzyme was then purified by gel filtration on Sephacryl S300.

The data in Tables II and III regarding experiments with various concentrations of ammonium sulfate for fractionation is included for the convenience of others who may work with the enzyme. Since the two different enzymes present are not differentiated by the synthetic substrate all data refer to total Azyme.

Gel filtration on Sephacryl S-300 has been used next (Fig. 4). Typical gel filtration results are 199.4 fold purification with 74.6% recovery (Experiment 28-3) and 462 fold purification with 40% recovery (Experiment 28-11).
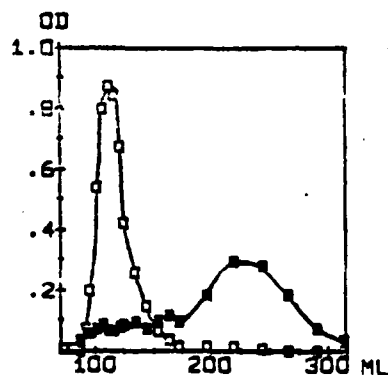

Fig. 4   Gel Filtration of Clostridial Enzyme Fractions (4.7 ml) were collected from a 2.5x80 cm column of Sephacryl S300 loaded with a 5 ml sample of ammonium sulfate precipitated enzyme and analyzed for Azyme (open squares) and protein (solid squares).

Table II Fractionation with Ammonium Sulfate

AMS fraction	Purfication	Specific activity*	% Recovery
Starting	–	0.19	–
0–40%	1.8	0.34	21.9
40%–60%	2.4	0.45	11.8
60%–80%	5.8	1.1	69.1
0–55%	15.8	3.0	15.8
55%–80%	24.7	4.7	24.7
0–55%	13.2	2.5	13.2
55%–85%	23.1	4.4	23.1
0–60%	34.2	6.5	34.2
60%–80%	10.5	2.0	10.5
0–60%	31.6	6.0	31.6
60%–85%	7.4	1.4	7.4
Original	–	0.36	–
0–40%	4.85	1.1	3.5
40%–52%	9.5	1.8	10.5
52%–60%	23.	4.9	24.3
60%–70%	26.	4.2	25.9
70%–80%	17.9	0.29	2.0

*Specific activity is in milliunits/mg protein

Table III Cultures with Cotton Plugs vs Tightly Capped Cultures

AMS	Specific activity	Total units
Cotton plug		
0–60%[a]	1.1×10^{-3}	0.20
60%–80%[a]	9.3×10^{-3}	0.22
80%–90%[a]	0.12×10^{-3}	0.03
0–90%[a]	1.7×10^{-3}	0.47
90%–100%[a]	0.23×10^{-3}	0.03
0–90%[b]	3.3×10^{-3}	0.13
0–90%[c]	3.2×10^{-3}	0.21
0–90%[d]	1.3×10^{-3}	0.07
Tight cap		
0–60%	2.6×10^{-3}	0.47
60%–80%	0.96×10^{-3}	0.18
80%–90%	0.098×10^{-3}	0.03

a, b, c and are different batches

Fractionation with cold ethanol has been a useful procedure to reduce the volume of material and remove most of the colored material. For this the filtrate was titrated to pH 6.0 with 1 N NaOH (about 1 ml/1) and cooled in an ice bath. Ethanol (95%, 0.4 volume) was cooled in a dry ice-ethanol bath and added slowly, keeping the temperature of the mixture at its freezing point. This step was carried out in a −18° cold room and the final solution was kept at its freezing point for 1–2 hr before centrifugation at −14°. The sparse

precipitate was washed once with 1/20 its volume of diluted ethanol (0.4 volume of 95% ethanol per volume of water). The precipitate was dissolved in ice cold water and lyophilized or dissolved in 2 M NaCl, dialyzed overnight at -5° and then against the buffer used for the enzyme assays. Although the overall yields have been only 18-32%, the enzyme is almost entirely of the type which converts red cells. Assuming equal amounts of the two enzymes are present in the filtrate would double the yield. Higher yields have been found when dithiothreitol (0.1 mM) was used. At the 10 liter level the cost of the ethanol procedure is only 15% as much as ammonium sulfate. The specific activity was 0.04 u/mg. In contrast to material prepared by ammonium sulfate fractionation, little additional purification was obtained by subsequent gel filtration.

Affinity purification of Clostridial Azyme

The Azyme was affinity purified at 4° on a 1X6 cm column, equilibrated with citrate phosphate buffer (0.355 g citric acid, 0.94 g Na_2PO_4 per liter titrated to pH 6.20). The sample, (0.49 U), was dialyzed against the buffer and applied at a flow rate of 3 ml/hr. The affinity column was washed with 39 ml of the citrate-phosphate buffer and the enzyme was eluted with a 20 ml linear salt gradient going to 0.5 M NaCl in the citrate-phosphate buffer followed by a 20 ml step gradient to 0.4 M galactose in the buffer containing 0.5 M NaCl (Fig. 5). In the experiment shown, 82% of the applied activity was recovered. The specific activity of the first peak was 0.06 u/mg and that of the second was 0.42 u/mg. In other experiments a 40 ml linear salt gradient was used.

> The enzyme was sufficiently pure that impurities in the galactose contributed the major part of the 280 nm reading in early experiments. In a typical case 65% of the optical density was from the galactose. Different lots of galactose gave similar high readings. One 0.4 M solution of galactose (Difco 396876) read 0.335. A similar concentration of sugar from Sigma (Lot 62F-0572) had an absorbance of 0.240. Ultra-filtration through an Amicon UM10 membrane (molecular weight cutoff 10,000) did not alter the absorbance suggesting that the impurity had a fairly low molecular weight. A solution passed through a 1x10 cm column of mixed bed ion exchange resin (Biorad AG 11A8) had an unchanged absorbance indicating that the impurity was uncharged. Batch treatment with charcoal (Norit) reduced the readings by 80%. A 1x21 cm column of 50-200 mesh charcoal which had been washed with boiling HCl, water, methanol and again with water and dried at 110° was prepared. Galactose, 0.8 M, was passed through the column at a flow rate of 12 ml/hr. The
> · absorbance was reduced to less than 0.02 i.e. less than 3% of the original. The galactose was recovered by freeze drying and used for subsequent experiments.

In most experiments the first peak come off about one column volume after the salt gradient was started. In other runs the specific activity of the second peak was as high as 1.03 u/mg with 43% of the applied activity appearing in the peak. Unfortunately the enzyme from the second peak did not convert A cells to O while that from the first peak did. (See page 17 for details.) Accordingly attention was focused on the affinity purification of the first peak. Variations in pH and salt gradient did not provide appreciable additional purification. Clostridial enzyme prepared by ethanol fractiontion gave a different elution pattern. A 40 ml salt gradient was used so that the first

enzyme appears later. The elution and monitoring were continued to a total volume of 100 ml. No second enzyme was found (Fig. 6). In this experiment 0.49 unit of ethanol fractionated enzyme, specific activity 0.01 u/mg was used. The Azyme had a specific activity of 0.03 u/mg and 73% was recovered.
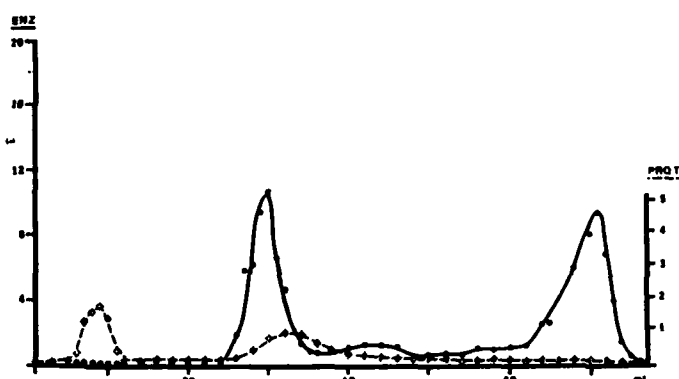
Fig. 5 Affinity Purification of the Clostridial Azyme from the Gel Filtration Step Protein (o--o) and Azyme (•—•)are plotted against the volume of eluate. Other conditions are described in the text.
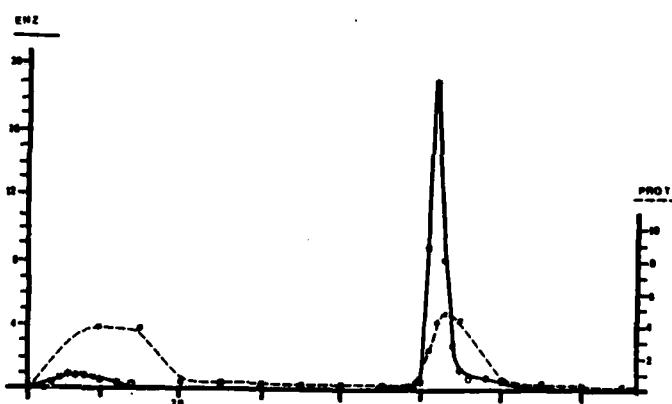
Fig. 6 Affinity Purification of Clostridial Azyme Prepared by Ethanol Fractiontion. Protein (*- -*) and Azyme (□—□) are plotted against volume of eluate.

Sialidase content of clostridial Azyme

The sialidase content of the Azyme from Clostridium paraputrificum was examined. The partially purified enzyme appears to be sialidase free. This supports the findings of Paoni et al. The purity of the preparations and difficulties with the assay are important factors. The thiobarbituric acid method was used. Aminoff and Paoni also used this method. Paoni found no detectable sialidase in the partially purified material. Neither do we. Four spectra from the assay indicate one of the problems with the assay (Fig 7). In A the spectrum of the product of Clostridium perfringens sialidase action on fetuin (-----) is compared with a sialic acid standard (———) and a blank (·········). The spectra are quite similar. In B the products formed by the action of a filtrate from a Clostridium paraputrificum culture (-----) are compared with the sialic acid spectrum (———) and a blank which contained no fetuin (·········). The filtrate contains some colored substance which is clearly not sialic acid. But it is difficult to rule out a small amount of sialic acid. The worst case estimate, assuming that all of the optical density formed at 549 nm is due to sialic acid release, would be .088 units of

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sialidase per unit of Azyme. Frame C shows comparable data for material
recovered by gel filtration. There is no discernable sialidase. We believe
that there are less than 4×10^{-5} units per unit of Azyme. Frame D gives data
with affinity purified enzyme. While there is a small difference between
control (·······) and Azyme (————), the lack of any peak at 549 nm suggests
that again little sialic acid is present. Less Azyme was used in the
experiment so that the top limit is .0023 units per unit of Azyme. After
ammonium sulfate purification, gel filtration and DEAE purification, Aminoff
had 0.77 units per unit of Azyme from Clostridium perfringens.
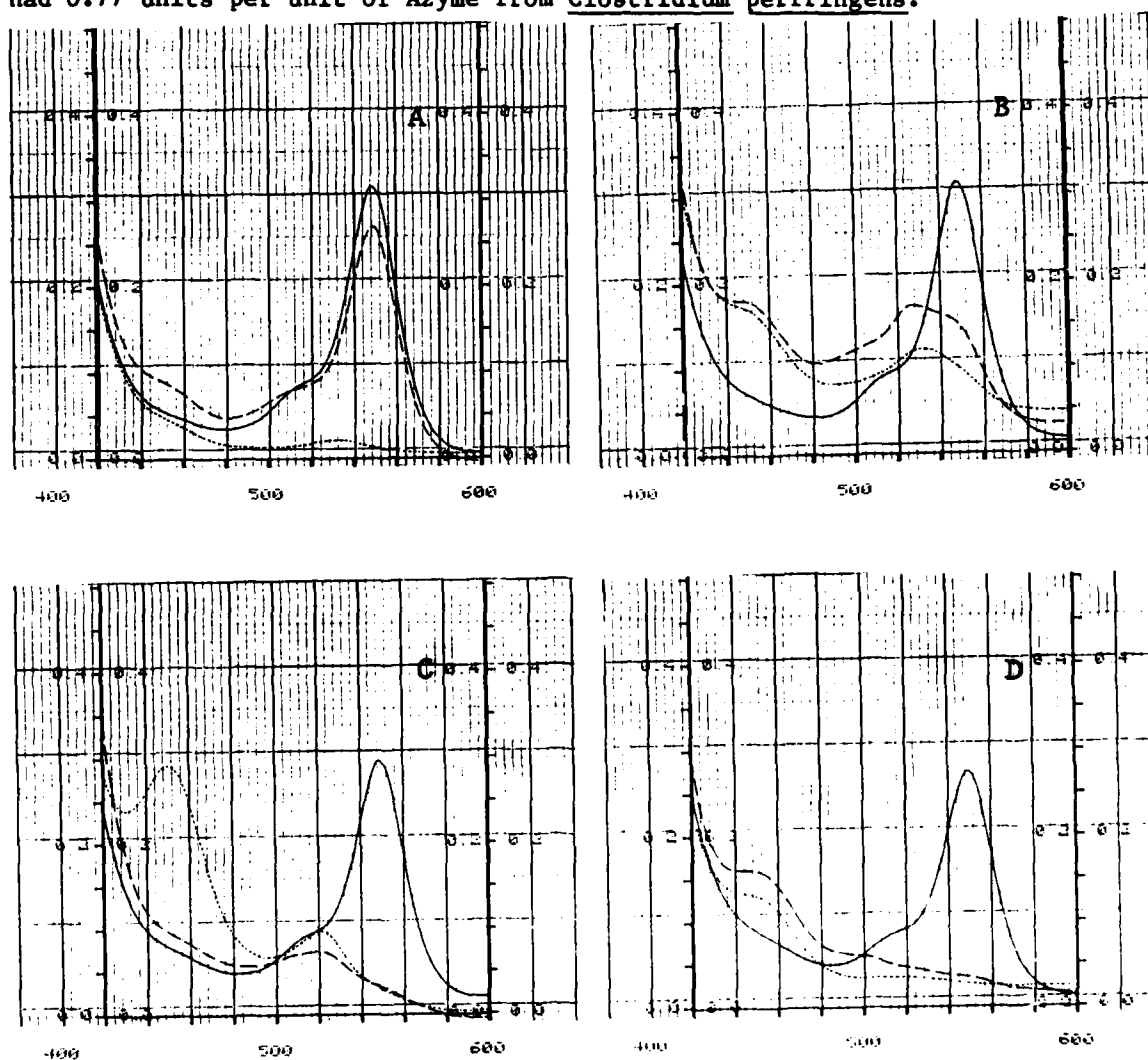
Fig. 7 Sialidase Assays on Clostridial Azyme

The absorbance is plotted against the wavelength. The thiobarbiturate assay
was used with sialidase from Clostridium perfringens (A), with culture
filtrate from Clostridium paraputrificum (B), with the latter enzyme purified
by gel filtration (C) and affinity chromatography (D). The solid lines are
sialic acid standards, the dashed lines are the assays and the dotted lines
are controls which contained no substrate.

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Properties of Azyme from Clostridium paraputrificum

A number of the kinetic and enzymatic properties of this Azyme were examined
by Sunita Sarin a senior at Kalamazoo College as part of a senior project.
She was not supported by ONR funds. Portions of the work were done to confirm
the findings of Paoni et al and to assure that the same enzyme is being
examined. All were done with the synthetic substrate p-nitrophenyl-2-acet-
amido-2-deoxy-α-D-galactopyranoside (Koch-Light).

pH optimum

The pH optimum of the partially purified enzyme prepared by ammonium sulfate
precipitation and gel filtration was determined at 37° using a series of 0.01M
buffers between pH 4 and 9. Sodium acetate buffer was used at pHs acid to 5.8
and Tris acetate was used above pH 7.8. Sodium phosphate buffers were used for
the other pHs. As indicated in Fig. 8, the optimal pH was between pH 6.0 and
6.5.
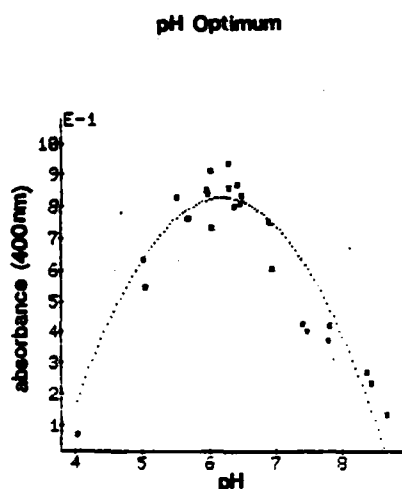
pH Optimum

Fig. 8   pH Optimum for Clostridial
Azyme
The activity is plotted aginst the pH.
A portion of the scatter is due to the
fact that a number of experiments done
at different times have been plotted.
Other details are given in the text.

Kinetic parameters at various temperatures

The velocity was determined at a series of temperatures using substrate
concentrations of 0.64, 0.91, 1.60 and 3.20 mM at pH 7 in 0.01M sodium
phosphate buffer containing 0.1 mM dithiothreitol and 0.68 mM $CaCl_2$.

Temperature	Km	Vmax
17°	.19	6.69×10^{-8}
23°	.30	1.46×10^{-7}
30°	.62	2.55×10^{-7}
37°	.88	2.73×10^{-7}
44°	1.14	5.66×10^{-7}

An Arrhenius plot of this data gave an energy of activation of 12,580
cal/mole.

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Calcium requirement

Enzyme assays under the above conditions were done at 3.20 mM substrate at calcium levels between 0 and 3.4 mM. Samples incubated overnight in 1 mM EDTA and ran in its presence had the same activity as those done in the absence of Ca^{++}. The activity was increased about 50% by the calcium (Fig. 9).
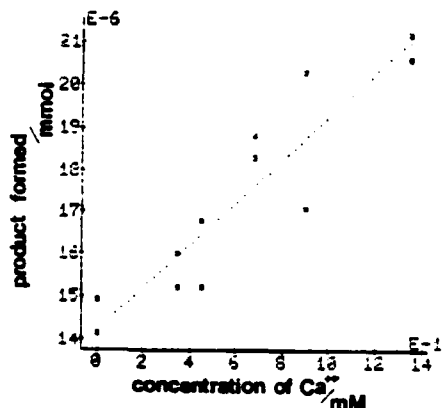

Fig. 9 Effect of Ca^{++} Concentration on Azyme Activity
Enzyme activity is plotted against Ca^{++} concentration

Requirement for reducing agent

Assays were done in the absence of DTT and in the presence of concentrations between 0.033 and 0.165 mM. DTT had little effect upon the activity. A small increase, about 10%, was observed at the highest DTT concentrations. These studies concern the assay conditions. Further studies would be needed regarding the role of DTT in stability during storage.

Stability studies

The partially purified enzyme was stored at 4° at a series of pHs between 4 and 10. Neither DTT nor calcium was added. The enzyme decayed rapidly at pH 10. The pH 4 points were less active than the rest. There was little loss of activity even after 13 days of storage (Fig. 10). At 37° the enzyme was less stable. The best stability was observed at pHs between 5 and 6 (Fig. 11). The reactions were in 0.02% sodium azide. The stability of affinity purified Azyme and crude material prepared by gel filtration was compared at 4° and at 37° at pH 6 in the absence of DTT and Ca^{++} (Fig. 12). The enzyme appeared fairly stable at 4° but about half of the activity was lost in 12 hr at 37°. It remains to be determined whether Ca^{++}, protein and/or DTT would stabilize the activity. Bovine serum albumin (0.1%) added to the assay of the affinity purified enzyme caused a 17% increase in activity suggesting that it might be useful in increasing the enzyme stability.
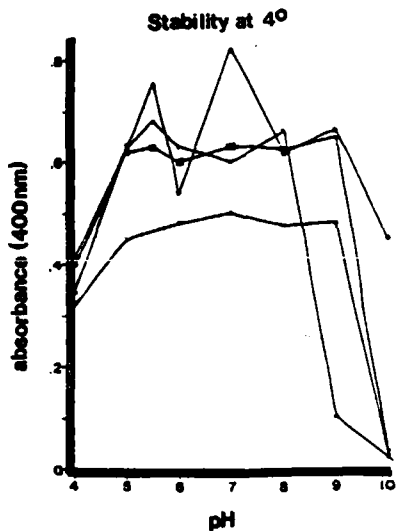
Stability at 4°

Stability at 37°

Fig. 10 Stability of Gel Filtered Azyme at 4°
Azyme purified by gel filtration was stored for 0 hr (●), 47.5 hr
(O), 143.5 hr (□) and 311 hr (■) at various pHs in the absence of
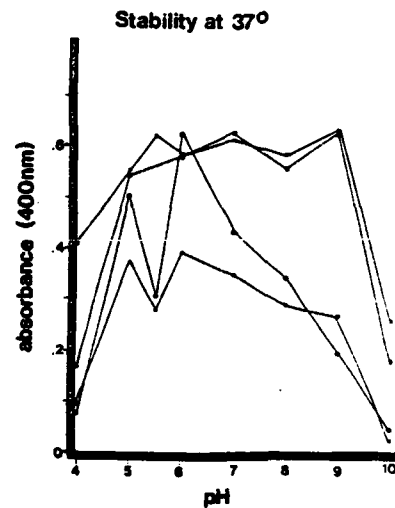added Ca^{++} and DTT. The absorbance is plotted against the pH.

Fig. 11 Stability of Gel Filtered Azyme at 37°
Conditions as in Fig 8 except temperature and times, 0 hr (■),
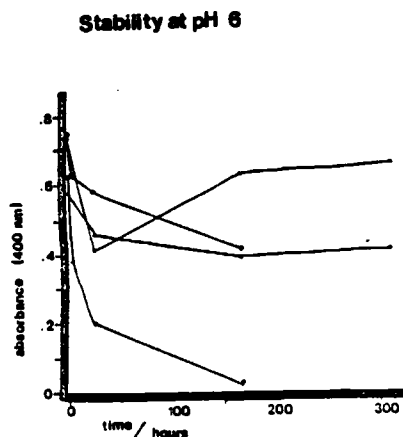2.3 hr (O), 47.5 hr (●) and 144 hr (□).

Stability at pH 6

Fig. 12 Stability of Affinity Purified
and Gel Purified Azyme
The reactivity of samples of
clostridial enzymes after various times
of storage. Squares represent data
from gel filtered enzyme and circles
that from the affinity purified
material. Solid symbols are from 4° and
open ones from 37°.

Determination of K_I for N-acetylgalactosamine
      The apparent Km was determined at 5 N-acetylgalactosamine concentrations
between 2 and 50 mM at 37° and the K_I calculated. The K_I was 1.38 mM. If one
calculates the maximum amount of end product formed during a typical red cell
conversion experiment, it is several orders of magnitude below the K_I. This
suggests that end product inhibition plays no part in the slowing observed as
the red cell conversion proceeds.

Effect of Hg^{++} on enzyme activity
      As found by Paoni, Hg^{++} is a very effective inhibitor of the
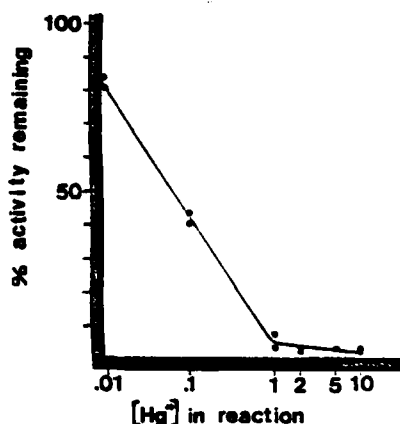clostridial Azyme (Fig. 13).

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Fig. 13 Effect of Hg^{++} on Clostridial Azyme Activity

Affinity purification of placental Azyme

Several experiments were done with the new batch of affinity matrix to establish its use at an earlier stage in the purification of placental Azyme. Initial experiments with a 0.5x5 cm (1ml) column give consistently satisfactory results (Fig. 14). A 2.5 ml sample of DEAE purified material which had been freeze dried was dissolved in a citrate phosphate buffer (citric acid 21.81g, disodium phosphate 28.45g /4 liters, pH 4.7). A suspension of dried enzyme was dialyzed overnight, centrifuged and applied in a volume of 2.5 ml at a flow rate of 3 ml/hr. The column was washed with 70 ml of the buffer prior to sample application and eluted with a 7 ml linear gradient going from 0 to 0.5 M NaCl in the buffer after a 7 ml wash with the initial buffer. In the experiment shown, 44% (0.15 U) of the applied activity was recovered in the main peak. The purification was 346 fold. Performing the affinity step at an earlier stage would probably eliminate the tedious step with hydroxylapatite.
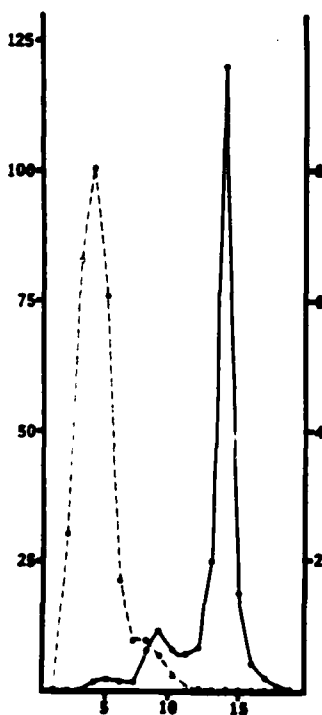

Fig. 14 Affinity Purification of DEAE Purified Placental Enzyme
Azyme activity (———) and protein (-----) are plotted against fraction number. Fraction volume 1.2 ml. Initial load 0.34 U. Other conditions as described in the text.

Conversion of Type A Red Cells to Type O with clostridial enzyme

The Azyme from Clostidium paraputrificum removes A activity from type A red cells (Fig. 15). The rate of loss is related to enzyme concentration. One of the unexpected findings was that the removal leveled off after a period of time. This was not due to destruction of the enzyme. After 48 hrs at 37° followed by 2.5 days at room temperature most of the original activity remained. We then considered that erythrocyte metabolism (ie. the formation of lactic acid from glucose) might acidify the reaction beyond its optimal pH. This too was found not to be the case. The initial pH used was 6.80 and the final pH after the time above was 6.79 ± 0.02 in 9 determinations. We then considered that the end product might be inhibiting the reaction. N-acetyl-galactosamine proved to be a good inhibitor but is probably not the cause of the leveling off of the reaction. Calculation of the amount expected to be liberated in relation to the K_I suggestes that this is not the problem. Failure to increase the rate of reaction by replacing the enzyme solution with fresh solution further suggests that this is not the cause.
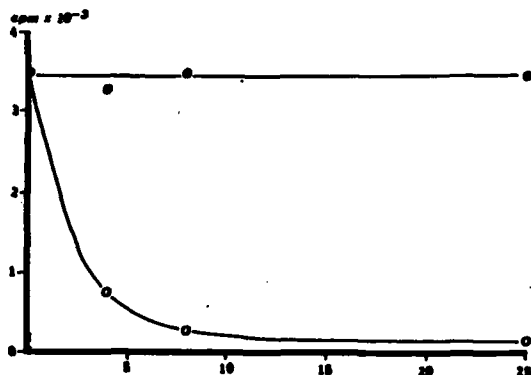

Fig. 15 Effect of Affinity Purified Clostridial Azyme Peak I on Type A Red Cells
The reaction mixture (O) contained 80ul of packed red cells and 80 ul of affinity purified clostridial Azyme (0.08 units)(uM/min) in phosphate (25 mM) buffered saline pH 6.8 and $CaCl_2$. The cells from 2.5ul of reaction mixture were used for each assay. Solid circles have buffer in place of enzyme. Controls with O cells (not shown) averaged 210 cpm. The second peak from the column was inactive.

Three minor problems were encountered in getting the conversion to occur. All involved hemolysis. The reaction mixture described by Paoni recommends that 1/6 of the reaction mixture be 7mM $CaCl_2$. Addition of sufficient NaCl to bring its osmolarity to 300 mOsm greatly reduced hemolysis. In experiments with crude ammonium sulfate fractions, the Azyme itself was slightly hemolytic. This tendency which was mild disappeared after gel filtration of the enzyme. It is not clear whether the problem was associated with a hemolysin or with substances in the medium. At the suggestion of a colleague, the stability of the cells was further improved by the addition of glucose to the reactions. The final concentration of glucose was 37.5 mM.

Conversion of Type A Red Blood Cells with Azyme from Human Placenta

The Azyme from human placenta also removes A determinants from erythrocytes (Fig. 16). A number of experiments were done and the rate of removal appeared proportional to the amount of Azyme used. In the experiment shown about 99% of the A activity was removed. A human source was developed to avoid possible problems with immune reactions to Azymes from other species. Concerns with the possibility of viral contamination of human products would suggest that cloning the gene for the enzyme would be the best way to proceed. Although genes expressed in other organisms lack the glycoprotein portion of the normal protein, the enzymes produced are usually effective. A portion of the preparation used was given to Dr. Jack Goldstein at the New York Blood Center

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to see if it would remove the traces of A activity remaining after treatment of red cells with Azyme from chicken liver. The placental Azyme did not remove the residual A activity. One possibility is that it is due to a branched determinant. Galactosidases from <u>Bacteroides</u> <u>fragilis</u> and <u>Escherichia</u> <u>freundii</u> are known to attack linear but not branched determinants (Scudder et al, 1984).
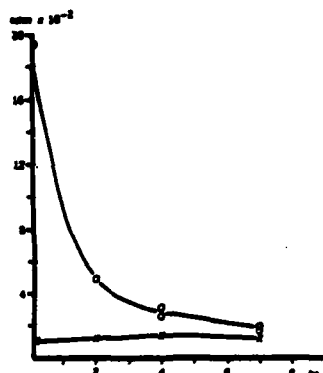

Fig. 16 Effect of Placental Azyme on Type A Red Blood Cells
A 50% red cell suspension was used with 2 ul of affinity purified placental Azyme (or buffer in the control).
0——0 = A cells + Azyme
X——X = 0 cells + Buffer

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Purification of α-N-Acetylgalactosaminidases by Affinity Chromatography
Thomas Kontry and Ray K. Brown (1984) Fed. Proc. 43, 1705. Abstract presented at the joint meeting of the American Society of Biological Chemists and the American Association of Immunologists St. Louis, MO June 3-6, 1984.

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